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Myringotomy in the *Junbo* mouse model of chronic otitis media alleviates inflammation and cellular hypoxia

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Nothing to declare

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Abstract

Objective
Ventilation of the chronically inflamed middle ear is a key outcome in functional middle ear surgery. Grommets eliminate middle ear effusion, but there is also evidence that they downregulate inflammation. The reason for this is not understood, but there is little to suggest alteration in Eustachian tube ventilatory capacity. Previous work has shown that the *Junbo* mouse model of chronic otitis media has hypoxic middle ear mucosa and bulla fluid leucocytes. Here we explore if surgical ventilation may alleviate chronic otitis media through downregulation of hypoxia.

Study Design
Surgical intervention on a mouse model of disease.

Methods
We established patency of myringotomy incision as 5 days in wild-type mice. We performed unilateral myringotomy on three cohorts of mice: 10 wild type controls, 12 *Junbo* mice, and 15 *Junbo* mice with additional removal of middle ear effusion. A small cohort of these mice were labeled in-vivo by intraperitoneal injection of pimodinazole to identify tissue hypoxia. Tissues were assessed for mucoperiosteal thickening and pimodinazole labeling, comparing operated to non-operated ears.

Results
Ventilation of the inflamed *Junbo* middle ear revealed significant reduction in inflammatory thickening associated with loss of pimodinazole labeling, suggesting resolution of cellular hypoxia.

Conclusion
Surgical ventilation may achieve therapeutic effect through alleviation of cellular hypoxia in the chronically inflamed middle ear. Targeted molecular therapy of hypoxia signaling may offer future alternative therapy for chronic OM.

Key words:
Otitis media
Grommets
Ventilation
Hypoxia
Junbo mouse model

Level of Evidence
N/A

Introduction

The middle ear evolved when our evolutionary predecessors moved from an aquatic to a terrestrial existence. The development of a gas-filled pocket next to the cochlea overcame air-fluid impedance mismatch, and so enabled audition of air-borne sound[1]. In mammals maintenance of this gas pocket is primarily achieved through gaseous exchange across the mucosal epithelium of the postero-superior middle ear cleft[2]. The Eustachian tube can also help to equilibrate gross pressure alterations, but likely plays a small role in minute-to-minute ventilation of the middle ear[3].

Chronic middle ear inflammation can lead to sub-atmospheric middle ear pressure, tympanic membrane retraction, and/or middle ear effusion, and thus disable the middle ear gas pocket. Re-establishment of middle ear ventilation is critical to the success of functional middle ear surgery[4-6]. The most common operation for restoration of hearing is the insertion of a grommet (ventilation tube), which reliably eliminates effusion in glue ear (chronic otitis media with effusion, COME). The therapeutic effect of this procedure is thought to be rheological, whereby creation of a vent reduces fluid inertia, allowing effusion to be cleared down the Eustachian tube by the ciliary apparatus of the protympanum. Aspiration of effusion at the time of grommet insertion is not necessary[7].

However, a number of lines of evidence suggest that grommets moderate the inflammatory process in addition to, or in place of, any rheological effect that may occur. Performing myringotomy and aspiration without intubation of the tympanic membrane leads to a rapid reaccumulation of effusion in children with COME[8]. Recurrence of effusion still occurs in 20-30% of children once grommets extrude[9-12], but retrospective case series suggest that the risk of recurrence is inversely related to the duration of ventilation[13 14]. There is also

endoscopic[15] and histological[16 17] evidence that resolution of middle ear inflammation is proportional to the duration of ventilation.

We have previously suggested that some, or even the main therapeutic effect of middle ear ventilation may be through alleviation of tissue hypoxia[18]. Hypoxia is a common finding in chronically inflamed microenvironments[19 20], coordinated through the transcription factor hypoxia inducible factor (HIF). Our group has explored the role of tissue hypoxia in the chronically inflamed middle ear by exploitation of the genetically altered *Junbo*[21] and *Jeff*[22] mouse models of chronic otitis media[18]. We have shown that systemic administration of pimodinazole (PIMO, a marker of tissues with an oxygen tension below 10 torr[23]) labels leucocytes in the exudate of the inflamed middle ear of both *Junbo* and *Jeff* mice, and also the middle ear mucosa in the *Junbo* mouse. Fluorescence-activated cell analysis confirms hypoxia in viable and apoptotic polymorphonuclear cells in the effusion, and transcriptome and proteome analysis of effusion reveals upregulation of a number of HIF responsive genes, notably in the signaling protein vascular endothelial growth factor (VEGF), a key executor of HIF response.

Here we use the *Junbo* mouse model to investigate our hypothesis that the medium to long-term beneficial effect of middle ear ventilation may be through alleviation of cellular hypoxia in the chronically inflamed middle ear. Specifically, we demonstrate that surgical myringotomy leads to reduction of mucoperiosteal inflammatory thickening, and loss of PIMO labelling in operated ears.

Materials and Methods

Mice

Wild type (WT +/+) and heterozygote *Junbo* (*Jbo/+)* mice (hereafter referred to as *Junbo* mice) on a congenic C3H/HeH background were generated and maintained as previously described. *Junbo* phenotype is characterised by the spontaneous development of chronic inflammatory disease that is anatomically restricted to the ME[18].

Establishing duration of myringotomy patency in the mouse

Surgical myringotomy has not previously been reported in the mouse. We performed myringotomy on WT C3H/HeH mice (aged 6-9 weeks) to establish normal duration of patency. Mice were anaesthetized by intraperitoneal injection of 10mg/kg xylazine and 100mg/kg ketamine. The posterior pars tensa of the left ear was incised using a disposable myringotome (Exmoor plastics, UK) under direct vision with an operating microscope. We always operated on the left ear because this was technically easier for our right-handed surgeon. Anaesthetic was reversed with 5mg/kg atipamezole hydrochloride. Mice were subsequently euthanized with an overdose of intraperitoneal barbiturate at time points of 3, 5, 7, and 10 days post-operatively (three mice for each time point). The tympanic membranes were assessed using a binocular microscope.

Myringotomy in three mouse cohorts

We subsequently operated on the left ear of three cohorts of mice, all aged 6-10 wks-old:

a) 10 WT controls who underwent myringotomy only
b) 12 *Junbo* mice who underwent myringotomy only
c) 15 *Junbo* mice who underwent myringotomy and removal of effusion using absorbent endodontic paper points.

The right ear was left unoperated to act as a control. Prior to operation all *Junbo* mice were assessed for bilateral visible evidence of tympanic membrane opacity, which we have previously shown to be a reliable marker of inflammatory effusion in the murine middle ear[24].

At intervals after myringotomy groups of mice were euthanized and skinned heads were fixed for 48 hours in 10% neutral buffered formalin, decalcified with Immunocal (Decal Corp) for 72 hours, and embedded in paraffin wax. 3μm sections were stained with haematoxylin and eosin. Middle ear mucoperiosteal thickness was measured as previously described[24] and compared between the operated left ear and the unoperated right ear using a paired sample t-test.

Five days after left-sided unilateral myringotomy 3 WT and 3 *Junbo* mice were labeled for 3 hours *in vivo* by i.p. injection with 60 mg/kg pimonidazole (PIMO) dissolved in 100μl of sterile PBS. The mice were euthanized and 4μm histological sections of the middle ear were immunostained using anti-PIMO rabbit polyclonal PAb2627AP primary antibody at 1:200 dilution at room temperature for 60 mins (Hypoxprobe-1 Omni Kit; hpi Hypoxyprobe Inc., Burlington MA) followed by goat-anti rabbit IgG HRP conjugate secondary antibody at 1:50 dilution at room temperature for 30 mins (Dako P0448) according to the manufacturer's instructions and with the following modifications. Antigen retrieval was by incubation at 60^{O}C overnight in Vector high pH buffer (Vector Laboratories H-3301) to prevent detachment of tissue from the electrostatically charged slide; both primary and secondary antibodies were diluted in Dako antibody diluent (S0809); and visualisation was carried out using Liquid DAB+ (Dako K34811).

Ethical approval

Full details of these studies were reviewed and approved by MRC Harwell ethical review committee. The humane care and use of mice in this study was carried out under the authority of the appropriate UK Home Office Project License.

Results

We established that myringotomy in WT mice was reliably patent at up to five days post-operatively (table 1) and used this time point in later experiments in *Junbo* mice. There were no significant complications or identifiable adverse effects from myringotomy. In each cohort data for mucoperiosteal thickness were normally distributed (Kolmogorov-Smirnov test).

In WT mice (cohort a, n=10) there was no evidence that myringotomy induced mucosal inflammation. The difference in mucoperiosteal thickness between left operated (19.9 +/- 2.1 μm: mean and standard error of mean) and right unoperated (16 +/- 1.5 μm) ears in WT mice was not significant (figure 1). There was no visible effusion in any of these ears.

In Junbo mice (cohort b, n=12) there was histological evidence of reduced middle ear inflammation in response to myringotomy, although effects were variable. Effusion volume was difficult to quantify precisely. The cellularity of bulla fluid varied and histological processing resulted in uneven shrinkage of bulla fluid profiles. Nevertheless the qualitative impression was of reduced effusion in the operated compared to the unoperated ear, and in many cases appeared to have resolved completely. Mucoperiosteal thickness in this cohort was less in the left operated ear (62.1 +/-9.1 μm) than the right unoperated ear (87.1 +/-9.1 μm), $p<0.05$ (figure 1).

In Junbo mice that underwent myringotomy with fluid removal (cohort c, n=15) we again found a variable response to surgery on qualitative histological assessment. However, in the majority of cases (12/15 cases) there was no evidence of reappearance of effusion in the operated ear after bulla fluid removal at time of surgery, and there was a reduction in

inflammation, sometimes markedly so (figure 2). Mean mucoperiosteal thickness in the left operated ear was significantly less (84.6 +/- 9.3 μm) than in the right unoperated ear (104.6 +/- 8.3 μm), $p<0.05$ (figure 1).

When data for cohort b and c were combined (i.e. all n=27 mice undergoing myringotomy with or without removal of effusion, analysed as a group) the mean mucoperiosteal thickening in the operated ear was significantly less (75.0 +/- 6.9 μm) compared to the unoperated ear (97.1 +/- 6.3μm), $p<0.003$ (figure 1).

In the three WT mice PIMO labeling was only present in the healing myringotomy site, a feature also seen in the *Junbo* mice (figure 3). Two of three *Junbo* mice showed PIMO staining for hypoxia in the unoperated ear, labeling both middle ear mucoperiosteum and bulla exudate macrophages. In both of these cases in the contralateral operated ear there was very little effusion and no PIMO labeling of the mucoperiosteum (figure 3). The other *Junbo* mouse had persistent effusion in both ears despite myringotomy, and here the macrophages in the effusion showed bilateral PIMO staining (but without PIMO labeling of the mucoperiosteum).

Discussion

Macroscopic otitis media in *Junbo* heterozygote mice is evident from the accumulation of middle ear bulla fluids resulting in tympanic membrane opacity. In (*n*=54) 8-wk-old *Junbo* mice the incidence of bilateral fluids was 78%, unilateral fluids 13% and there was no overt bulla fluid in either ear of 9% of mice[18].

In the current experiments *Junbo* mice were assessed pre-operatively for the presence of bilateral fluids. The important assumptions in our experimental design were that myringotomy itself did not induce middle ear inflammation and bulla fluid effusion; that *Junbo* mice had pre-operative bilateral middle ear inflammation (and the bulla effusion was sufficiently cellular to drive middle ear hypoxia); and that myringotomy with or without fluid removal would significantly reduce bulla effusion volume and cellularity.

We have shown here that myringotomy did not itself induce middle ear bulla effusion or mucoperiosteal thickening in WT mice but there was a proliferative epithelial repair response at the site of myringotomy incision. There was a statistically significant reduction (~23%) in inflammatory thickening of the mucoperiosteum in the *Junbo* mouse five days after surgical myringotomy (with or without removal of effusion), the period in which the incision is patent. We also found that myringotomy alone resulted in a qualitative reduction in the space occupied by bulla effusion, and that myringotomy with fluid removal resulted in a 80% reduction in the occurrence of bulla fluid five days post surgery. The results of a small-scale *in vivo* labeling experiment with PIMO to identify sites of tissue and cellular hypoxia provide preliminary evidence that mucoperiosteal labeling was reduced in two of three operated *Junbo* middle ear bullae. In these cases mucoperiosteal thickening was reduced and effusion was almost entirely removed. In

the third *Junbo* mouse effusion was present in both the operated and unoperated ear, and here PIMO labeling of the effusion was present bilaterally.

These data are consistent with our hypothesis that myringotomy reduces middle ear mucoperiosteum inflammation and leads to resolution of bulla effusion, and that an important biological mechanism of middle ear ventilation may be through alleviation of cellular hypoxia in chronically inflamed middle ear tissues. However the reduction of mucoperiosteal thickening and resolution of effusion was not universal after myringtomy and it is possible that more prolonged ventilation could improve the response to surgery in the *Junbo* mouse model.

In the non-inflamed middle ear the partial pressures of oxygen, carbon dioxide, and nitrogen mirror those of venous blood, as a result of trans-mucosal gaseous exchange[25-28]. In our mouse models the chronically inflamed middle ear is hypoxic[18] and human data also supports hypoxia pathway activation in COME, with elevated levels of VEGF reported in two studies[29 30].

Hypoxia is a common finding in inflamed environments. Inflammation increases cellular energy demands, but simultaneously distances inflammatory cells from blood vessels due to cellular oedema and mucosal hyperplasia and extravasation of leucocytes into the bulla lumen. Transcriptional regulation through HIF-VEGF pathways acts to compensate for the hypoxic environment, and restore tissue homeostasis to enable cellular survival under stress. However, persistent hypoxia signaling is known to be maladaptive, and can contribute to ongoing inflammation and tissue damage[31 32].

Grommets expose the middle ear space to the relative hyperoxia[33] of atmospheric oxygen, and this would presumably reverse tissue hypoxia, both of cells in the effusion and ultimately mucosal cells. This may reduce inflammation and hence lead to the eventual resolution of middle ear effusion. However, it is interesting to note that hypoxia pathways also have direct transcriptional activity on mucin production. The *MUC5AC* gene, which encodes one of the major mucins found in COME[34], contains a highly conserved HIF binding site that acts as a transcriptional promoter. Experimental disruption of this binding site abolishes stimulated mucin secretion[35]. Middle ear ventilation may therefore downregulate hypoxia pathways, which in turn eliminates one major driver for the transcription of the mucins that are the hallmark feature of COME.

Alleviation of hypoxia may be an important adjunct to rheological effect of grommets. It is noteworthy that grommets have not been shown to affect the ventilatory function of the Eustachian tube in the short[36], medium[15 37 38], or long-term[39], and so a purely physical action of grommets would seem an inadequate explanation as to why prolonged ventilation affects subsequent disease severity or recurrence.

In this study we have used the *Junbo*[21] mouse model of chronic otitis media. The *Junbo* mouse spontaneously develops a highly penetrant chronic otitis media by 28 days of age, with a neutrophil and macrophage rich effusion. *Junbo* carries a point mutation at the *Mecom* locus (*also known as the Mds1-Evi1* cluster), which may affect its interaction with TGF-ß[40 41] JNK[42] or NF-κB[43] pathways. Mouse models have made a considerable contribution to the experimental investigation of otitis media, because of their easy husbandry and the repertoire of techniques available to manipulate their genome, leading

to the recovery of several mouse models of chronic otitis media (reviewed elsewhere[44 45]). However mouse models do have limitations[46], including species differences in inflammatory response, which could limit the applicability of these models to human disease. Nevertheless, hypoxia pathway activation has been reported as a feature of human COME[29 30], and this compels us to believe that the *Junbo* mouse model is valid for exploration of human pathobiology.

An extension of our study may be to look at the biological effects of a more extended duration of ventilation. This could be achieved by laser myringotomy[47-54] or by application of mitomycin-C[55-59] to the incision, but these methods probably only slightly prolong patency of myringotomy and may in themselves contribute to inflammation.

Conclusion

We have undertaken the first animal study to investigate the biological effects of ventilation in chronic otitis media. Using the *Junbo* mouse model, we have shown that surgical ventilation reduces inflammatory thickening of the middle ear mucoperiosteum, and that this may be due to alleviation of tissue hypoxia in the middle ear. Induction of chronic otitis media in larger species through bacterial challenge or genetic engineering may enable our studies to be repeated and extended to include intubation of the tympanic membrane.

Grommets are the only treatment known to reliably lead to resolution of effusion in COME[11]. If, as we propose, their therapeutic effect is through alleviation of tissue hypoxia, it suggests that in the future their therapeutic benefit could be replaced with targeted molecular therapy based on hypoxia pathways. Indeed, targeting hypoxia pathways with VEGF

receptor inhibitors moderates hearing loss in our mouse models of chronic otitis media[18]. The findings presented here suggest this may be a fruitful avenue to pursue in man, in place of surgical ventilation.

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References

1. Christensen-Dalsgaard J, Carr CE. Evolution of a sensory novelty: tympanic ears and the associated neural processing. *Brain Res Bull* 2008;75(2-4):365-70.
2. Ars B, Wuyts F, Van de Heyning P, Miled I, Bogers J, Van Marck E. Histomorphometric study of the normal middle ear mucosa. Preliminary results supporting the gas-exchange function in the postero-superior part of the middle ear cleft. *Acta oto-laryngologica* 1997;117(5):704-7.
3. Sade J, Cinamon U, Ar A, Seifert A. Gas flow into and within the middle ear. *Otol Neurotol* 2004;25(5):649-52.
4. Haginomori S, Takamaki A, Nonaka R, Mineharu A, Kanazawa A, Takenaka H. Postoperative aeration in the middle ear and hearing outcome after canal wall down tympanoplasty with soft-wall reconstruction for cholesteatoma. *Otol Neurotol* 2009;30(4):478-83.
5. Ikeda M, Yoshida S, Ikui A, Shigihara S. Canal wall down tympanoplasty with canal reconstruction for middle-ear cholesteatoma: post-operative hearing, cholesteatoma recurrence, and status of re-aeration of reconstructed middle-ear cavity. *The Journal of laryngology and otology* 2003;117(4):249-55.
6. Shinnabe A, Hara M, Hasegawa M, Matsuzawa S, Kodama K, Kanazawa H, et al. Relationship between postoperative aeration around the stapes and postoperative hearing outcome after canal wall down tympanoplasty with canal reconstruction for cholesteatoma. *Otol Neurotol* 2011;32(8):1230-3.
7. Laina V, Pothier DD. Should we aspirate middle-ear effusions prior to insertion of ventilation tubes? *The Journal of laryngology and otology* 2006;120(10):818-21.
8. Mandel EM, Rockette HE, Bluestone CD, Paradise JL, Nozza RJ. Efficacy of myringotomy with and without tympanostomy tubes for chronic otitis media with effusion. *The Pediatric infectious disease journal* 1992;11(4):270-7.
9. Gates GA, Avery CA, Prihoda TJ, Cooper JC, Jr. Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion. *N Engl J Med* 1987;317(23):1444-51.
10. Maw R, Wilks J, Harvey I, Peters TJ, Golding J. Early surgery compared with watchful waiting for glue ear and effect on language development in preschool children: a randomised trial. *Lancet* 1999;353(9157):960-3.
11. Browning GG, Rovers MM, Williamson I, Lous J, Burton MJ. Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. *Cochrane Database Syst Rev* 2010(10):CD001801.
12. Boston M, McCook J, Burke B, Derkay C. Incidence of and risk factors for additional tympanostomy tube insertion in children. *Archives of otolaryngology--head & neck surgery* 2003;129(3):293-6.
13. Yaman H, Yilmaz S, Guclu E, Subasi B, Alkan N, Ozturk O. Otitis media with effusion: recurrence after tympanostomy tube extrusion. *International journal of pediatric otorhinolaryngology* 2010;74(3):271-4.
14. Ahn JH, Yoon TH, Pae KH, Kim TS, Chung JW, Lee KS. Clinical manifestations and risk factors of children receiving triple ventilating tube insertions for treatment of recurrent otitis media with effusion. *Pediatrics* 2006;117(6):e1119-23.
15. Takahashi H, Honjo I, Fujita A, Kurata K. Transtympanic endoscopic findings in patients with otitis media with effusion. *Archives of otolaryngology--head & neck surgery* 1990;116(10):1186-9.
16. Takahashi H, Sando I. Histopathology of tubotympanum of children with otitis media treated with ventilation tubes. *The Annals of otology, rhinology, and laryngology* 1992;101(10):841-7.

17. Kiroglu F, Kaya M, Ozsahinoglu C, Soylu L, Polat S. Changes of middle ear mucosa in secretory otitis media treated with ventilation tubes. *Acta oto-laryngologica* 1990;110(3-4):266-73.
18. Cheeseman MT, Tyrer HE, Williams D, Hough TA, Pathak P, Romero MR, et al. HIF-VEGF Pathways Are Critical for Chronic Otitis Media in Junbo and Jeff Mouse Mutants. *PLoS genetics* 2011;7(10):e1002336.
19. Dehne N, Brune B. HIF-1 in the inflammatory microenvironment. *Exp Cell Res* 2009;315(11):1791-7.
20. Frede S, Berchner-Pfannschmidt U, Fandrey J. Regulation of hypoxia-inducible factors during inflammation. *Methods Enzymol* 2007;435:405-19.
21. Parkinson N, Hardisty-Hughes RE, Tateossian H, Tsai HT, Brooker D, Morse S, et al. Mutation at the Evi1 locus in Junbo mice causes susceptibility to otitis media. *PLoS genetics* 2006;2(10):e149.
22. Hardisty-Hughes RE, Tateossian H, Morse SA, Romero MR, Middleton A, Tymowska-Lalanne Z, et al. A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. *Human molecular genetics* 2006;15(22):3273-9.
23. Kizaka-Kondoh S, Konse-Nagasawa H. Significance of nitroimidazole compounds and hypoxia-inducible factor-1 for imaging tumor hypoxia. *Cancer Sci* 2009;100(8):1366-73.
24. Bhutta MF, Hedge EA, Parker A, Cheeseman MT, Brown SD. Oto-endoscopy: a reliable and validated technique for phenotyping otitis media in the mouse. *Hearing research* 2011;272(1-2):5-12.
25. Doyle WJ, Seroky JT. Middle ear gas exchange in rhesus monkeys. *The Annals of otology, rhinology, and laryngology* 1994;103(8 Pt 1):636-45.
26. Hamada Y, Utahashi H, Aoki K. Physiological gas exchange in the middle ear cavity. *International journal of pediatric otorhinolaryngology* 2002;64(1):41-9.
27. Sade J, Ar A. Middle ear and auditory tube: middle ear clearance, gas exchange, and pressure regulation. *Otolaryngol Head Neck Surg* 1997;116(4):499-524.
28. Kania RE. Trans-mucosal gas exchange in normal and pathological conditions. In: Ars B, editor. *Chronic Otitis Media*. Amsterdam: Kugler Publications, 2008.
29. Jung HH, Kim MW, Lee JH, Kim YT, Kim NH, Chang BA, et al. Expression of vascular endothelial growth factor in otitis media. *Acta oto-laryngologica* 1999;119(7):801-8.
30. Sekiyama K, Ohori J, Matsune S, Kurono Y. The role of vascular endothelial growth factor in pediatric otitis media with effusion. *Auris, nasus, larynx* 2011;38(3):319-24.
31. Ikeda E. Cellular response to tissue hypoxia and its involvement in disease progression. *Pathol Int* 2005;55(10):603-10.
32. Eltzschig HK, Carmeliet P. Hypoxia and inflammation. *N Engl J Med* 2011;364(7):656-65.
33. Felding JU, Rasmussen JB, Lildholdt T. Gas composition of the normal and the ventilated middle ear cavity. *Scand J Clin Lab Invest Suppl* 1987;186:31-41.
34. Kerschner JE, Tripathi S, Khampang P, Papsin BC. MUC5AC expression in human middle ear epithelium of patients with otitis media. *Archives of otolaryngology--head & neck surgery* 2010;136(8):819-24.
35. Young HW, Williams OW, Chandra D, Bellinghausen LK, Perez G, Suarez A, et al. Central role of Muc5ac expression in mucous metaplasia and its regulation by conserved 5' elements. *Am J Respir Cell Mol Biol* 2007;37(3):273-90.
36. van der Avoort SJ, van Heerbeek N, Zielhuis GA, Cremers CW. Sonotubometry in children with otitis media with effusion before and after insertion of ventilation tubes. *Archives of otolaryngology--head & neck surgery* 2009;135(5):448-52.

37. van Heerbeek N, Ingels KJ, Snik AF, Zielhuis GA. Eustachian tube function in children after insertion of ventilation tubes. *The Annals of otology, rhinology, and laryngology* 2001;110(12):1141-6.
38. Straetemans M, van Heerbeek N, Schilder AG, Feuth T, Rijkers GT, Zielhuis GA. Eustachian tube function before recurrence of otitis media with effusion. *Archives of otolaryngology--head & neck surgery* 2005;131(2):118-23.
39. Caye-Thomasen P, Stangerup SE, Jorgensen G, Drozdziewic D, Bonding P, Tos M. Myringotomy versus ventilation tubes in secretory otitis media: eardrum pathology, hearing, and eustachian tube function 25 years after treatment. *Otol Neurotol* 2008;29(5):649-57.
40. Alliston T, Ko TC, Cao Y, Liang YY, Feng XH, Chang C, et al. Repression of bone morphogenetic protein and activin-inducible transcription by Evi-1. *The Journal of biological chemistry* 2005;280(25):24227-37.
41. Izutsu K, Kurokawa M, Imai Y, Maki K, Mitani K, Hirai H. The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling. *Blood* 2001;97(9):2815-22.
42. Kurokawa M, Mitani K, Yamagata T, Takahashi T, Izutsu K, Ogawa S, et al. The evi-1 oncoprotein inhibits c-Jun N-terminal kinase and prevents stress-induced cell death. *EMBO J* 2000;19(12):2958-68.
43. Xu X, Woo CH, Steere RR, Lee BC, Huang Y, Wu J, et al. EVI1 Acts as an Inducible Negative-Feedback Regulator of NF-kappaB by Inhibiting p65 Acetylation. *J Immunol* 2012;188(12):6371-80.
44. Rye MS, Bhutta MF, Cheeseman MT, Burgner D, Blackwell JM, Brown SD, et al. Unraveling the genetics of otitis media: from mouse to human and back again. *Mamm Genome* 2011;22(1-2):66-82.
45. Tyrer HE, Crompton M, Bhutta MF. What have we learned from murine models of otitis media? *Current allergy and asthma reports* 2013;13(5):501-11.
46. Bhutta MF. Mouse Models of Otitis Media: Strengths and Limitations. *Otolaryngol Head Neck Surg* 2012.
47. Zanetti D, Piccioni M, Nassif N, Campovecchi C, Redaelli de Zinis LO. Diode laser myringotomy for chronic otitis media with effusion in adults. *Otol Neurotol* 2005;26(1):12-8.
48. Poyrazoglu E, Cincik H, Gungor A, Gurpinar B, Yildirim S, Candan H. The effects of incisional myringotomy and CO2 laser myringotomy on rat tympanic membranes. *International journal of pediatric otorhinolaryngology* 2004;68(6):811-5.
49. Cotter CS, Kosko JR. Effectiveness of laser-assisted myringotomy for otitis media in children. *The Laryngoscope* 2004;114(3):486-9.
50. Deutsch ES, Cook SP, Shaha S, Brodsky L, Reilly JS. Duration of patency of laser-assisted tympanic membrane fenestration. *Archives of otolaryngology--head & neck surgery* 2003;129(8):825-8.
51. Sedlmaier B, Jivanjee A, Gutzler R, Huscher D, Jovanovic S. Ventilation time of the middle ear in otitis media with effusion (OME) after CO2 laser myringotomy. *The Laryngoscope* 2002;112(4):661-8.
52. Valtonen HJ, Poe DS, Shapshay SM. Experimental CO2 laser myringotomy. *Otolaryngol Head Neck Surg* 2001;125(3):161-5.
53. Silverstein H, Jackson LE, Rosenberg SI, Conlon WS. Pediatric laser-assisted tympanostomy. *The Laryngoscope* 2001;111(5):905-6.
54. Silverstein H, Kuhn J, Choo D, Krespi YP, Rosenberg SI, Rowan PT. Laser-assisted tympanostomy. *The Laryngoscope* 1996;106(9 Pt 1):1067-74.
55. Cincik H, Gungor A, Cekin E, Saglam O, Yildirim S, Poyrazoglu E, et al. Effects of topical application of mitomycin-C and 5-fluorouracil on myringotomy in rats. *Otol Neurotol* 2005;26(3):351-4.

56. Jassir D, Buchman CA, Gomez-Marin O. Safety and efficacy of topical mitomycin C in myringotomy patency. *Otolaryngol Head Neck Surg* 2001;124(4):368-73.

57. O'Reilly RC, Goldman SA, Widner SA, Cass SP. Creating a stable tympanic membrane perforation using mitomycin C. *Otolaryngol Head Neck Surg* 2001;124(1):40-5.

58. Estrem SA, Vanleeuwen RN. Use of mitomycin C for maintaining myringotomy patency. *Otolaryngol Head Neck Surg* 2000;122(1):8-10.

59. Estrem SA, Batra PS. Preventing myringotomy closure with topical mitomycin C in rats. *Otolaryngol Head Neck Surg* 1999;120(6):794-8.

Table 1:
Macroscopic patency of myringotomy in WT mice up to 10 days after incision. Unilateral surgery was performed on three mice for each time-point of two, five seven, and ten days post surgery.

Days post myringotomy	Proportion patent
2	3/3
5	3/3
7	1/3
10	0/3

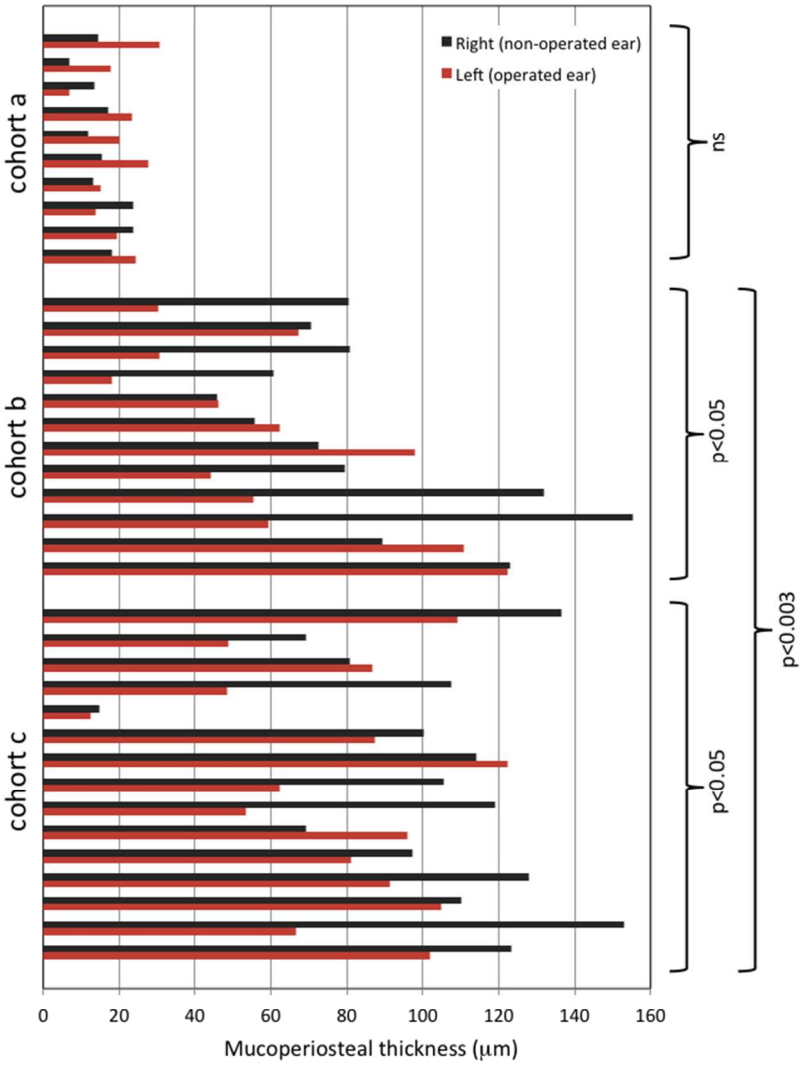
Figure 1:
Mucoperiosteal thickness in the operated (myringotomy) and non-operated ears of the mice: WT mice (cohort a), *Junbo* mice (cohort b) and *Junbo* mice treated with myringtomy and removal of effusion (cohort c). p-values refer to a paired sample t-test. ns = not significant.

Figure 2:
The histology of the middle ear of *Junbo* mice five days post myringotomy and removal of the bulla effusion.

(A) In the majority of cases there is minimal recurrence of effusion (e); (B) in a minority of cases myringotomy did not effectively reduce bulla effusion. Note thickening of tympanic membrane results from epithelial and stromal hyperplasia at the site of surgical myringotomy (s). A and B scale bar = 500 μm

Figure 3:
Hypoxia labeling with PIMO of the middle ear in the *Junbo* mouse is reduced five days post myringotomy and removal of effusion.

(A and C) In two *Junbo* mice the operated ear has much reduced effusion (e) and reduced inflammatory thickening of the mucoperiosteum (m) compared to (B and D) the corresponding contralateral unoperated ear where both the mucoperiosteum and leucocytes within the effusion are labeled with the hypoxia marker PIMO (arrows indicate cells with brown DAB staining).(E and F) The healing surgical myringotomy site is labeled with PIMO in both WT (E) and (F) *Junbo* mice. A-D scale bar = 100 μm; E and F scale bar = 200 μm.



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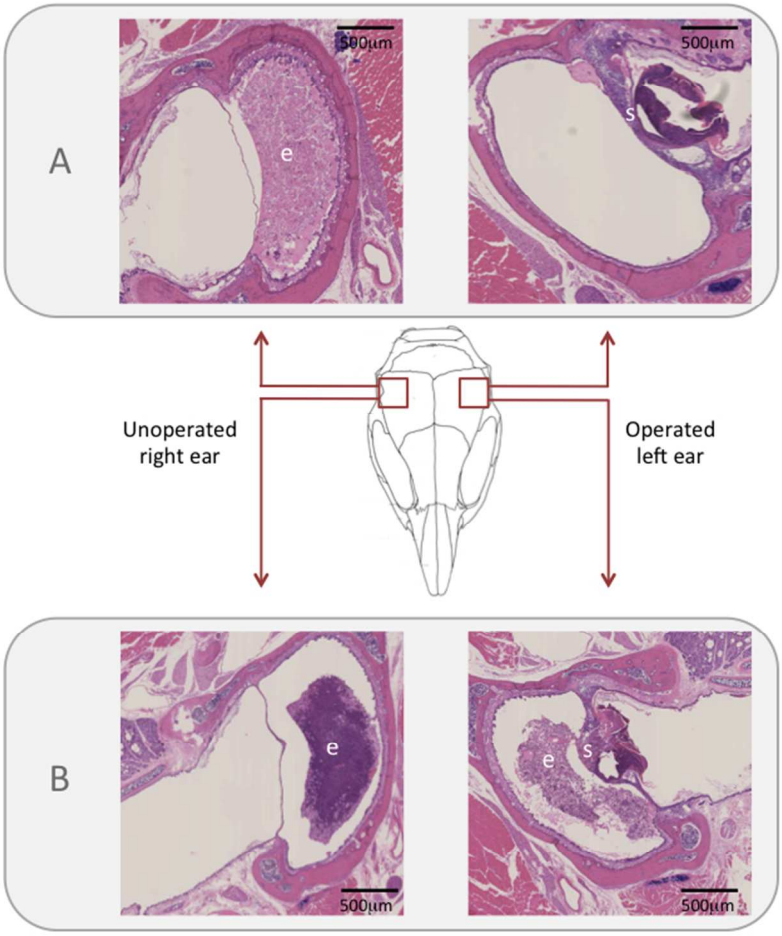

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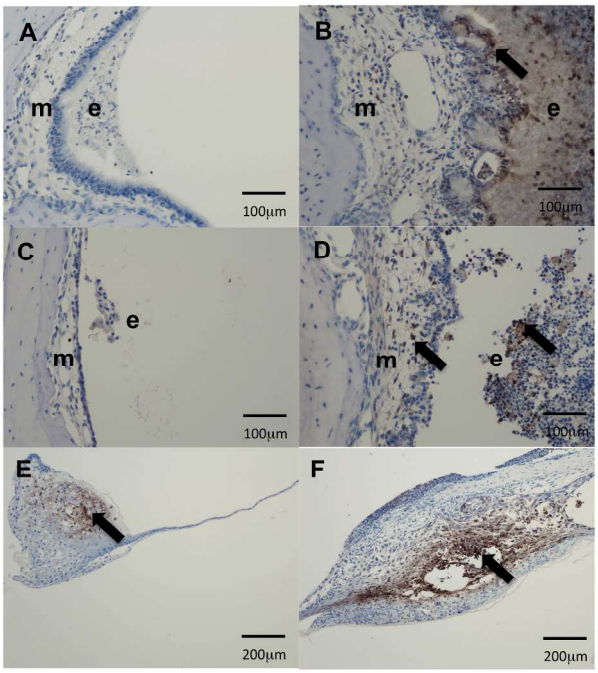

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